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Phenotype-driven strategies for screening *Candida parapsilosis* complex for molecular identification

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ABSTRACT

In this study, phenotypic methods presented >80% agreement with the molecular identification of 59 *Candida parapsilosis* complex. Growth at 15% NaCl or pH 7.0 significantly reduced cfu-counts of *Candida orthopsilosis*, suggesting these conditions may support the development of phenotypic methods for the differentiation of the cryptic species of *C. parapsilosis* complex.

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In 2005, Tavanti et al.[1] demonstrated that the yeast known as *Candida parapsilosis* was actually composed by three cryptic species, and proposed the current nomenclature: *C. parapsilosis sensu stricto*, *Candida orthopsilosis*, and *Candida metapsilosis*. Several phenotypic assays have been used to identify *C. parapsilosis* species complex,[2–4] but they do not accurately distinguish its cryptic species, thus requiring the use of molecular methods for differentiation. Thus, this study initially aimed to evaluate the efficacy of two phenotypical approaches to identify *C. parapsilosis sensu lato*, for further molecular identification of its cryptic species. Afterward, the growth kinetics

of strains of *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis* was assessed at different salinity and pH levels to investigate phenotypic differences between these cryptic species.

The 59 yeasts included in this study were isolated from human, animal, and food samples and were presumptively identified as *C. parapsilosis sensu lato*, by growth on chromogenic medium, micromophological analysis on conrmeal-Tween 80 agar and biochemical analysis based on urease production, carbohydrate assimilation and fermentation and nitrogen assimilation. These microorganisms belong

to the culture collection of the Specialized Medical Mycology Center of the Federal University of Ceará.

After storage, the identification of 34/59 isolates as *C. parapsilosis sensu lato* was confirmed by growth on chromogenic medium (CHROMagar™ *Candida* medium, BD™, Paris, France) and micromorphological analysis on cornmeal-Tween 80 agar, which revealed typical morphological features, *i.e.* branched pseudohyphae in a delicate tree-like pattern and giant cells, or features suggestive of *C. parapsilosis sensu lato*, such as wide elongated blastoconidia and short branched pseudohyphae.[5] The other 25 isolates were re-identified by growth on chromogenic medium and automated biochemical analysis by Vitek 2™ system (bioMérieux, Jacarepaguá, Rio de Janeiro, Brazil), which showed typical biochemical profile of *C. parapsilosis* or biochemical features with low discrimination profile, yielding equal probability of being two or three different species, including *C. parapsilosis sensu lato*.

Afterwards, these 59 isolates of *C. parapsilosis sensu lato* were submitted to molecular identification of their cryptic species, by restriction enzyme PCR assay of the *sadh* gene, using the enzyme *BanI*, as previously described.[1,6] The restriction patterns of *C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 96139, and a previously identified strain of *C. metapsilosis* [6] were used to confirm identification. Six isolates with cell arrangement suggestive of *C. parapsilosis sensu lato*, showed unspecific band pattern, after amplification of the *sadh* gene. Hence, they were submitted to DNA sequencing, according to Desnos-Ollivier et al.[7]

Finally, six strains of each cryptic species, including the reference strains *C. parapsilosis* ATCC 22019 and *C. orthopsilosis* ATCC 96139 (Table 1), were randomly selected for the growth kinetic assays, according to Lachance et al.[8] with some modifications. The strains were cultured on PDA at 35 °C for 48 h. Yeast inocula were prepared in sterile saline solution, with approximately 10^6 cfu/mL, using a Neubauer chamber. Aliquots of 400 µL were transferred to tubes containing 3600 µL of one of the tested media. Yeasts were gown in Sabouraud broth (SAB, Difco™, Franklin Lakes, New Jersey, USA), used as cell growth control, and SAB supplemented with 5%, 10%, or 15% NaCl (w/v), or in SAB at pH 3, 5, and 7. The tubes were incubated at 35 °C, and fungal growth was evaluated at 0, 24, 48, and 72 h by culturing onto PDA. The plates were incubated

Table 1 – Strains of *Candida parapsilosis* complex selected for the study of growth kinetics.

Origin	Strains	*Candida parapsilosis* complex
Human	CEMM 05-1-054	*C. parapsilosis sensu stricto*
	CEMM 01-1-165	*C. parapsilosis sensu stricto*
	CEMM 05-5-083	*C. orthopsilosis*
	CEMM 05-5-093	*C. orthopsilosis*
	CEMM 05-5-087	*C. metapsilosis*
	CEMM 05-5-086	*C. metapsilosis*
	CEMM 01-1-167	*C. metapsilosis*
Veterinary	CEMM 03-2-045	*C. parapsilosis sensu stricto*
	CEMM 01-1-196	*C. parapsilosis sensu stricto*
	CEMM 01-1-186	*C. parapsilosis sensu stricto*
	CEMM 01-1-204	*C. parapsilosis sensu stricto*
	CEMM 01-1-178	*C. orthopsilosis*
	CEMM 01-1-200	*C. orthopsilosis*
	CEMM 01-1-183	*C. orthopsilosis*
	CEMM 01-1-190	*C. orthopsilosis*
	CEMM 03-2-046	*C. metapsilosis*
	CEMM 01-1-199	*C. metapsilosis*
Food	CEMM 01-1-169	*C. metapsilosis*

at 35 °C for 48 h, and the number of cfu/mL was determined. Assays were conducted in triplicate at two different moments.

Growth kinetics of *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis* under different salinity and pH levels was analyzed, by calculating cfu-counts and average growth rate of each species: average growth rate (%) = [(number of living cells after 72 h − number of living cells at time zero)/number of living cells at time zero] × 100. Friedman and Kruskal–Wallis nonparametric tests were used to analyze each species, followed by Dunn's posthoc test. *p*-values < 5% indicated significant differences.

Out of the 34 isolates reassessed by growth on chromogenic agar and micromorphology, 28 showed a typical cellular arrangement of *C. parapsilosis* complex, including the presence of giant cells, which were further identified as *C. parapsilosis sensu stricto* (*n* = 16), *C. orthopsilosis* (*n* = 8), and *C. metapsilosis* (*n* = 4) (Fig. 1A), confirming the phenotypical identification of the species complex. Six isolates, on the other hand, had an arrangement similar to that of *C. parapsilosis sensu lato* but did not present giant cells (Table 2), showing atypical band patterns, after amplification of the *sadh* gene (Fig. 1B),
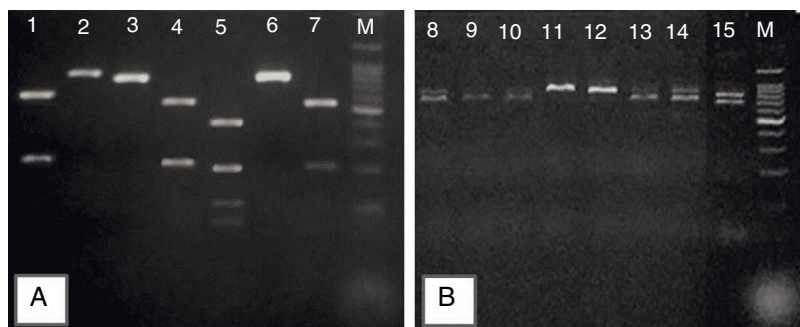
Fig. 1 – **Representative 2% agarose gel of *Candida parapsilosis sensu lato*, after specific PCR reactions. (A) Products obtained after the digestion of *sadh* gene – PCR fragments by the *BanI* restriction enzyme distinguishing the cryptic species: *C. parapsilosis sensu stricto* (lines 1, 4, 7); *C metapsilosis* (line 5); and *C. orthopsilosis* (lines 2, 3, 6); (B) the band patterns of 716 bp, obtained after *sadh* gene PCR reaction, suggestive of *C. parapsilosis sensu lato* (lines 11 and 12) and unspecific band patterns obtained for *Candida guilliermondii* (lines 8, 9, 10, 13, 14 and 15). Molecular marker: 1000 bp.**

| Table 2 – Presumptive phenotypical identification of isolates of *C. parapsilosis* species complex and molecular confirmation through PCR-REA. | | | | | | | | |
|---|---|---|---|---|---|---|---|
| Methods | Strains (*n*) | Chromogenic medium (*n*) | Phenotypical characteristics | | Phenotypical identification (*n*) | *n* | Molecular identification (*n*) | Phenotypical identification agreement *n* (%) |
| *Microscopic analyses* | | | | | | | | |
| Chromogenic agar, Dalmau culture | 34 | White (17), pale pink (11) | Giant-cells, branched pseudo-hyphae and elongated blastoconidia | | *C. parapsilosis* (28) | 28 | *C. parapsilosis sensu stricto* (16), *C. orthopsilosis* (8), *C. metapsilosis* (4) | 28/34 (82.3) |
| | | Pale pink (6) | Wide elongated blastoconidia, with reduced filamentation | | *C. parapsilosis* (6)[b] | 6 | – | |
| *Biochemical profile*[a] | | | | | | | | |
| Chromogenic agar, Vitek 2 | 25 | White (3), pale pink (17) | (−) dRAFa, IMLTa, XLTa, ARBa, dCELa, ERYa, GENa, ISBEa; (v)IARAa, GRTas | (+) TyrA, dGNTa, dGATa; (v)GRTas | *C. parapsilosis* (20) (99%)[c] | 20 | *C. parapsilosis sensu stricto* (17), *C. orthopsilosis* (2), *C. metapsilosis*(1) | 23/25 (92.0) |
| | | White (1), pale pink (3) | (−) TyrA, IMLTa, LACa, XLTa, ARBa, dCELa, ERYa, GGT; (v)dXYLa, dRAFa, dGATa, GENa, GRTas | (+) dRAFa, dGNTa, GENa, ISBEa, GRTas; (v)IMLTa, dGATa (LDO) | *C. famata/C. parapsilosis* (4) (LDO)[d] | 4 | *C. parapsilosis sensu stricto* (1), *C. orthopsilosis* (2) | |
| | | Pale pink (1) | (−) LysA, TyrA, IRHAa, IMLTa, NAGA1, XLTa, ARBa, dCELa; (v) dXYLa, dRAFa, IARAa, dGNTa, dGATa, GLYLa, GENa, AGLU, GRTas | (+) dRAFa, NAGa, ARG, SACa, ACEa, dGALa, dGATa, GENa, ISBEa, GRTas; (v)GLYLa, IMLTa, IARAa, dGNTa | *C. famata/C. parapsilosis C. tropicalis* (1) (LDO)[d] | 1 | – | |

[a] Biochemical profile (+), (−) and (v) distinct from each presumptive identification of the Vitek 2™ system. The variable biochemical profile corresponds to the profile of the strain (−) or (+) or contradictory to the given test. Biochemical tests: LysA, ʟ-lysine arylamidase; IMLTa, ʟ-malate; ARG, arginine; ERYa, erythritol; GLYLa, glycerol; TyrA, tyrosine arylamidase; ARBa, arbutin; dGALa, ᴅ-galactose; GENa, gentiobiose; LACa, lactose; dCELa, ᴅ-cellobiose; GGT, gamma-glutamyl-transferase; dRAFa, ᴅ-raffinose; NAGA1, PNP-N-acetyl-BD-galactosaminidase 1; dMEla, ᴅ-melibiose; ISBEa, ʟ-sorbose; IRHAa, ʟ-rhamnose; XLTa, xylitol; SACa, saccharose/sucrose; AGLU, alpha-glucosidase; dTREa, ᴅ-trehalose; IARAa, ʟ-arabinose; dGATa, ᴅ-galacturonate; dXYLa, ᴅ-xylose; ACEa, acetate; GRTas, glucuronate; NAGa, N-acetyl-glucosamine; dGNTa, ᴅ-gluconate.

[b] Micromorphologically, presented cell arrangement weakly suggestive of *C. parapsilosis* complex, without typical features, such as giant-cells and branched pseudohyphae in a delicate tree-like pattern, presented unspecific molecular profile and were identified as *C. guilliermondii* through DNA sequencing.

[c] Percentage of confidence in the identification by the Vitek 2™ system.

[d] Low discrimination organism.
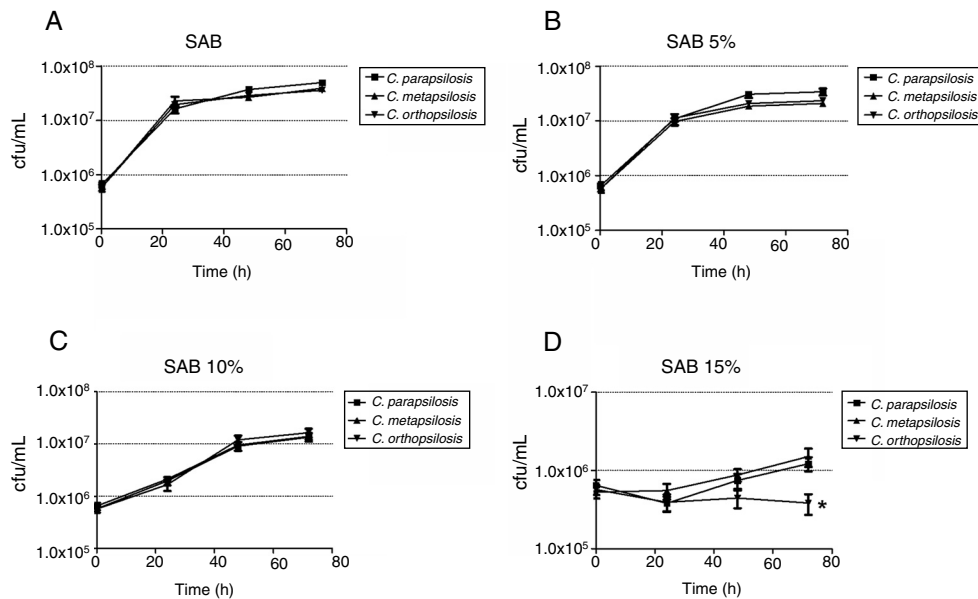
Fig. 2 – Growth kinetics, expressed as cfu/mL of the cryptic species of the *Candida parapsilosis* complex after growth up to 72 h of culture in media with different salinity levels. (A) Sabouraud broth without NaCl (growth control); (B) Sabouraud broth with 5% NaCl; (C) Sabouraud broth with 10% NaCl; (D) Sabouraud broth with 15% NaCl. *Indicates statistically significant differences.
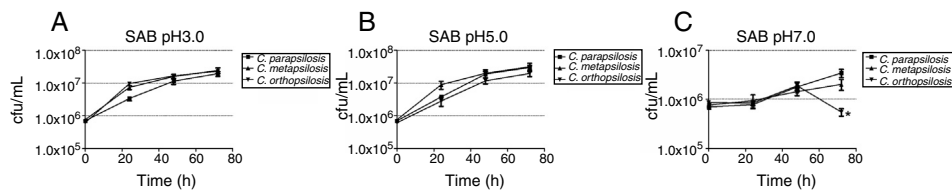
Fig. 3 – Growth kinetics, expressed as cfu/mL, of the cryptic species of the *Candida parapsilosis* complex, after growth up to 72 h of culture in media at different pH levels. (A) Sabouraud broth pH 3.0; (B) Sabouraud broth pH 5.0; (C) Sabouraud broth pH 7.0. *Indicates statistically significant differences.

and were identified as *Candida guilliermondii* by DNA sequencing. Hence, micromophological evaluation had an accuracy of 82.3% (28/34).

Among the 25 isolates identified by growth on chromogenic agar and automated biochemical analysis, 20 were confirmed as *C. parapsilosis sensu lato*, while five yielded inconclusive biochemical profiles, presenting equal probability of being *Candida famata* or *C. parapsilosis* ($n = 4$) or *C. famata*, *C. parapsilosis* or *Candida tropicalis* ($n = 1$) (Table 2). The molecular identification revealed that 23/25 (92%) isolates belonged to the *C. parapsilosis* complex (18 *C. parapsilosis sensu stricto*, 4 *C. orthopsilosis* and 1 *C. metapsilosis*) (Fig. 1A).

The growth kinetics at different salinities showed that maximum growth was achieved in SAB (control), and similar growth patterns were observed at 5% NaCl. The number of viable cells reduced ($p < 0.05$) at 10% and 15% NaCl for all cryptic species, especially *C. orthopsilosis*, which presented significantly ($p < 0.05$) lower average growth rate at 15% NaCl than the other two species (Fig. 2). Regarding pH levels (Fig. 3), similar growth patterns were observed at pH 3 and 5, but viable cells of the three species were significantly reduced ($p < 0.05$) at pH 7, especially for *C. orthopsilosis*, which presented lower

($p < 0.05$) average growth rate than *C. metapsilosis* and *C. parapsilosis sensu stricto*.

Recent studies have reported the reliability of conventional methods for the phenotypic identification of yeasts of medical importance, emphasizing that it is necessary to choose the best combination of methods to increase identification accuracy.[9,10]

Concerning micromorphological analysis, most strains (28/34) showed a typical morphology of *C. parapsilosis sensu lato*, and were correctly identified, corroborating the findings of a previous study.[3] However, 6/34 isolates presented a cellular arrangement similar to that of *C. parapsilosis sensu lato*, but without giant cells, and were further identified as *C. guilliermondii* by DNA sequencing. These results show that micromorphological identification is reliable for the identification of *C. parapsilosis* complex, before performing the molecular analyses, when typical features are observed, with emphasis on giant cells.

Regarding the automated biochemical analysis, 80% (20/25) of the tested isolates were confirmed as *C. parapsilosis sensu lato*, while the remaining five isolates presented inconclusive biochemical identification. Even though the use of Vitek

2[TM] system has been well established in clinical microbiology laboratories for yeast identification,[3,11–13] its accuracy for the identification of *C. parapsilosis lato sensu*, *C. famata*, *C. tropicalis* and *C. guilliermondii* has been considered low (approximately 90%), since low discrimination results are commonly obtained.[3,9–11,14] These species have similar biochemical profiles, as observed in the present study, impairing the analysis by Vitek 2[TM] system, which may not differentiate between them. Alternatively, the inclusion of micromorphological analysis in the identification procedures would likely solve this issue, once conventional phenotypical methods have shown to be highly effective for the identification of *C. parapsilosis sensu lato*.[3,10,11]

The observation of epidemiological variations between the cryptic species of the complex led to the report of differences in virulence among them, with *C. parapsilosis sensu stricto* and *C. orthopsilosis* being more virulent than *C. metapsilosis*.[15,16] Therefore, based on these epidemiological observations, phenotypic differences among the cryptic species of *C. parapsilosis* complex were pursued, in this study, in response to distinct growth conditions, such as salinity and pH levels. *C. orthopsilosis* was the least tolerant cryptic species to 15% NaCl and pH 7. These findings may explain, at least in part, the environmental distribution of the cryptic species of the *C. parapsilosis* complex, as they may show different phenotypic plasticity in response to distinct environmental conditions.[17] Moreover, the different growth patterns of *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis* in hypersaline and neutral pH media may be important for further differentiation of the cryptic species.

In conclusion, micromorphological analysis and automated biochemical analysis with Vitek 2[TM] system are reliable approaches for phenotypic screening of *C. parapsilosis* complex, especially when typical morphological features and biochemical profiles are observed. Concerning growth under different salinity and pH levels, *C. orthopsilosis* was the least tolerant cryptic species to 15% NaCl and pH 7. These findings may partly explain the ecological differences between these cryptic species and may also be important for further identification tests, since these features may support the development of phenotypic methods for differentiating the cryptic species of *C. parapsilosis* complex.

Ethical approval

This study assessed strains stored in the culture collection of the Specialized Medical Mycology Center of the Federal University of Ceará. The recovery of these strains was performed in previous studies carried out by our research group, which were approved by the Ethics Committee for the Use of Animals of the State University of Ceará.

Conflicts of interest

The authors declare no conflicts of interest.

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